Myocardial injury and performance in hypoxaemic neonates: Effects of oxygen and carbon dioxide during reoxygenation.

An experimental study in newborn pigs

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OM AT VILLE KUNNE

Der er mangt, man ikke kan
og af mange grunde,
men som det dog kun kom an
på at ville, for at man
ville kunne kunne.

Piet Hein

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Publications included in the thesis

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II: **Fugelseth D., Børke W.B., Lenes K., Matthews I., Saugstad O.D., Thaulow E.,** Restoration of cardio-pulmonary functions with 21% vs. 100% O_2 after hypoxaemia in newborn pigs.
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III. **Børke W.B., Munkeby B.H., Halvorsen B., Bjørnland K., Tunheim S.H., Borge G.I.A, Thaulow E., Saugstad O.D.,** Increased myocardial matrix metalloproteinases in hypoxemic newborn pigs during resuscitation. Effects of oxygen and carbon dioxide.
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IV: **Børke W.B., Edvardsen T., Fugelseth D., Lenes K., Ihlen H., Saugstad O.D., Thaulow E.,** Reduced left ventricular function in hypoxemic newborn pigs. A strain Doppler echocardiographic study.
Submitted

Selected abbreviations and definitions

CO	cardiac output
cTnI	cardiac troponin I
Hb	haemoglobin
hypoxaemia	deficient oxygenation of arterial blood
hypoxia	oxygen supply decreased relative to metabolic demand
FiO_2	fraction of inspired oxygen
LV	left ventricle
LVP	left ventricular pressure
MMP	matrix metalloproteinase
ORAC	oxygen radical absorbance capacity
PAP	pulmonary artery pressure
$PaCO_2$	arterial carbon dioxide tension
PEEP	positive end expiratory pressure
PVR	pulmonary vascular resistance
ROS	reactive oxygen species
RV	right ventricle
RVP	right ventricular pressure
SBP	systemic blood pressure
SDE	strain Doppler echocardiography
TDI	tissue Doppler imaging

Introduction

Definition of asphyxia

Asphyxia is a Greek term and means loss of pulse (1). There is no clear definition of the term, though perinatal asphyxia or birth asphyxia often is defined as impaired placental- or pulmonary oxygen delivery to the neonate. As a consequence the neonate is going through a period of hypoxaemia and hypercapnia with a subsequent respiratory and metabolic acidosis (1). In clinical work, the diagnosis of perinatal asphyxia predominantly is based on its consequences, and there is a general agreement that a combination of perinatal stress (i.e. fetal bradycardia, acidosis, low Apgar scores), multiple organ affection and neurological symptoms (i.e. hypoxic ischemic encephalopathy) are required for the diagnosis (2).

Cardiac abnormalities in perinatal asphyxia

Myocardial performance may vary subsequent to asphyxia. The cardiac dysfunction is often underdiagnosed and requires a high index of suspicion to detect (3;4). The clinical picture varies from brady- or tachycardia to cardiogenic shock (1;5).

Myocardial failure subsequent to asphyxia was first reported in 1961(6). The authors described a group of asphyxiated neonates with left ventricular (LV) heart failure within the first 24 hours of life. A decade later three term infants were reported with a syndrome of acute LV failure accompanied by cyanosis and pulmonary and systemic venous congestion (7). Rowe et al (8) classified the cardiac abnormalities following asphyxia in 1978, broadly as:

1) Transient myocardial ischaemia of the newborn

2) Transient mitral regurgitation of the newborn

3) Transient tricuspid regurgitation of the newborn

4) Persistent pulmonary hypertension of the newborn

Increased pulmonary artery pressure (PAP) and tricuspid regurgitation is the most common, and although the cardiac abnormalities were described as separate clinical entities, it is important to understand that the distinction may not be clear, and there is much overlap in the pathogenesis. In severely asphyxiated neonates post-mortem autopsies have showed ischemic papillary muscle necrosis and diffuse subendocardial left ventricular (LV) necrosis secondary to perinatal asphyxia (9;10).

Aetiology

The aetiology of asphyxia in the newborn is multifactorial, including abruption or infarction of the placenta, excessive uterine contraction, fetal or maternal bleeding, and compression of the umbilical cord. Infection is contributing to its severity, and the clinical picture in severe asphyxia may present similar to what is seen in severe congenital heart disease, thus it is mandatory to evaluate these neonates by echocardiography to exclude congenital heart disease.

Myocardium in the newborn

At birth the haemodynamic situation changes rather abruptly from a dominating high pressure right ventricular (RV) and low pressure LV system to an adult system with low pressure RV and high pressure LV (11;12). The RV is dominating in the fetal myocardium (11;13), and the

RV stroke volume is greater than of LV (14-16). It is thought that RV performs the majority of ventricular work during fetal life (11;12), and RV end-diastolic volume is larger than in LV. The RV dimensions are larger, and the curvature of the RV free wall is greater than of LV (17;18). Consequently, at birth RV systolic wall stress is greater than in LV, with a greater sensitivity to increased afterload of the RV (19). The myocardial work performed by left and right ventricle is changing oppositely after birth (20). LV work increases due to the enhanced LV stroke volume, systemic blood pressure (SBP), wall tension, and increased systemic vascular resistance, whereas RV work falls in association with the decrease in pulmonary vascular resistance (PVR) and PAP (21).

The growth of LV and RV is different postnatally (22). The circulatory changes at birth affect ventricular size, though the total dimension of the heart stays constant. LV weight and wall thickness increase relatively to body weight, whereas RV weight and wall thickness decrease over the first two days of life (23), eventually resulting in an equalization of ventricular size postnatal (24;25). The myocardium is thought to grow by cell division in fetal life, and by cell growth postnatally (26). The fetal RV myocytes are larger than the LV myocytes and the capillary lumen is greater (27). The differences in LV and RV myocytes reverse postnatal, as the LV work load increases (20).

In the myocytes, troponins and tropomyosin constitute the regulatory contractile protein of the thin filament of muscle myofibrils, which regulate the calcium-concentration-dependent force development of the myofilaments. The troponin complex exists in a group of three subunits, troponin I (cTnI), troponin T and troponin C. Troponin T binds the troponin complex to tropomyosin. Troponin I and C are located at the troponin T carboxyl terminus. CTnI is the actomyosin ATPase inhibiting subunit. There are however, several isoforms of the troponins.

Slow skeletal muscle troponin I is synthesized in the fetal myocardium, and the isoform is persistent until 9 months of age (28).

Until a certain degree the Frank-Starling mechanism characterised by the greater preload, the greater force of contraction, is found in the fetal and neonatal heart as well as the adult heart (29). The fetal and neonatal myocardium, however, is thought to work near the top of the Frank-Starling curve close to the "break point". The neonatal myocardium can only increase stroke volume to a small degree in response to an increase in preload (30;31). The sarcomere length and the extent of overlap between the thin and thick filament during contraction is crucial to the process of force development and contraction. It is, however, not the length itself, but the spacing between actin and myosin filaments that sets the level of Ca^{++} sensitivity (32;33).

In an animal model, it has been shown that oxygen consumption is approximately 40% higher in the neonatal compared to fetal and adult myocardium (34). The neonatal myocardium has greater myocardial glycogen stores (35;36), and glucose and lactate is thought to be the primary source metabolized in the immature myocardium (34). In the fetus this may be due to a deficiency in the enzyme carnitin palmitoyl transferase-*I*, responsible for transporting long-chain fatty acids into the mitochondria (37). Glucose ameliorates the effect of prolonged hypoxaemia in both fetal and adult heart (36).

Circulation in the newborn

At birth the circulatory system has been changed from a parallel to a serial circuit, and the gas exchange has been transferred from the placenta to the lungs. An adequate pulmonary circulation has been established, and the three fetal intra- and extra-cardial shunts; *ductus*

arteriosus, foramen ovale, and *ductus venosus* are gradually closed (38). The cardiovascular transition at birth has been instituted by the pulmonary physical expansion, ventilation, and increased oxygen tension subsequent to the first breath (39;40). The factors promoting vasoconstriction have been downregulated while the vasodilating mechanisms have become instantly operative. Accordingly, the pulmonary arteries and veins have been relaxed in response to endothelin-derived nitric oxide and dilatory prostaglandins, assisting the fall in pulmonary vascular resistance (PVR) (41). Pulmonary blood flow has rapidly been increased and the umbilical-placental circulation discontinued, gradually resulting in a circulation with equal left and right ventricular output in series.

At the onset of breathing, PVR decreases with a concomitant increase in pulmonary blood flow (39;42;43). Accordingly, as the pulmonary venous return increases, left atrial pressure exceeded right atrial pressure and foramen ovale is being functionally closed. By the first 24 hours of age the mean pulmonary artery pressure (PAP) has fallen below the mean SBP in most human infants, and by three days mean PAP in healthy human infants has been shown to be less than 50 % of the mean systemic blood pressure (SBP) (44;45). The rearrangement of circulation with closure of the fetal shunts contributes to the increase in LV preload and thus stroke volume. RV systolic pressure decreases while LV systolic pressure increases. Both ventilation, oxygenation, and umbilical cord occlusion contributes to a progressive shift in the LV and RV outputs (39;46). With the cessation of the placental circulation, the large placental run-off has been interrupted, with a concomitant increased systemic vascular resistance. LV output has been markedly enhanced and RV output increased to a smaller extent (15;16;47;48) equalising the LV output as the foramen ovale and ductus arteriosus have been closed (40;42).

Hypoxaemia-reoxygenation injury

In general, myocardial reoxygenation/reperfusion injury subsequent to hypoxaemia and/or ischaemia includes cardiac contractile dysfunction (49), arrhythmias (50) and reversible or irreversible myocyte damage including both apoptotic and necrotic cell death (51). The myocardial injury is associated with release of reactive oxygen species (ROS) and inflammation (52). In the hypoxaemic newborn, however, both physiological as well as cellular- and molecular mechanisms have to be addressed.

Hypoxic pulmonary vasoconstriction

The pulmonary circulation constricts in response to acute hypoxaemia, which is reversible on reexposure to oxygen (53;54). Severe pulmonary vasoconstriction as well as relaxation of ductus arteriosus are induced by the hypoxanthine-xanthine oxydase system forming ROS (55;56), and there has been found a relation between low alveolar oxygen tension and increased PVR (57). In the newborn, these haemodynamic changes following hypoxaemia have to be considered and kept in mind during tissue analyses and assessments of myocardial performance (58;59). The pulmonary vasculature is regulated by different mechanisms, including nitric oxide (NO) derived from the endothelium, reactive oxygen species generated by mitochondria, changes in intracellular K^+, Ca^{2+}, and H^+ concentrations as well as prostacyclin, prostaglandin E_2, and bradykinin (53;60;61). Despite extensive characterization of the structural and functional changes occurring in pulmonary vasculature in response to hypoxaemia, the exact cellular mechanisms underlying hypoxaemic pulmonary vasoconstriction remain poorly understood (62).

ROS

There is growing evidence that oxidative stress and ROS formation are involved in hypoxaemia/reoxygenation/reperfusion injury (63-65), and antioxidant treatment has been shown to protect myocardium against reperfusion injury (66). ROS are reactive redox intermediates produced during the sequential reduction of molecular oxygen. ROS fall into two groups; those that contain unpaired electrons (O_2^-, OH^-), or those that have the ability to extract electrons from other molecules (H_2O_2, HOCl). These species may damage biomolecules directly, or initiate chain reactions in which ROS are passed from one molecule to another, resulting in extensive damage to cell structures such as membranes and proteins (67). A rapid and transient burst of huge amounts of ROS are described with the first moments of reperfusion (68). ROS may be generated as a result of high oxygen concentration (69-71). Paradoxically, there are also described protective effects of ROS, and biological tissues have substantial ability to tolerate ROS under normal conditions (68;72). Living organisms have not only adapted to protect against ROS, they have also developed mechanisms for the beneficial uses of free radicals (68). ROS can be preventive as signal preconditioning protection, and induce stress responses that lead to survival (72).

In the setting of hypoxaemia/ischaemia and subsequent reoxygenation/reperfusion, however, the antioxidant defences are eroded and this carefully orchestrated homeostasis is altered (68;73). Tissues have several endogenous ROS scavenger systems to combat the cytotoxic actions of ROS, including enzymes as superoxide dismutases, gluthation peroxidases, and catalases, large molecules as albumin, small molecules as ascorbic acid, glutathtion, uric acid, and carotenoids (74). During pathological conditions as hypoxia and acidosis, tissues have increasing capability of generating the destructive hydroxyl radical ($^{\cdot}OH$). Hydroxyl radicals are extremely reactive and may cause direct cell membrane damage, lipid peroxidation, and

damage to proteins and sulfhydryl bonds (68). ROS is associated with inflammation and cytokine release by upregulation of matrix metalloproteinases (75-77), hence ROS act as mediator of cell injury and myocardial dysfunction (63-65).

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) belong to a family of zinc and calcium dependent endopeptidases involved in varieties of physiological and pathological processes through the turnover of the extracellular matrix (78;79). There are four subgroups of MMPs, according to which components of the extracellular matrix they degrade, the collagenases (MMP-1, MMP-8 and MMP-13), the gelatinases (MMP-2 and MMP-9), the stromelysins (MMP-3, MMP-10 and MMP-11), and the membrane-type MMPs, which may both activate other MMPs and degrade various extracellular matrix components (65). MMPs are important in normal physiological processes as embryogenesis and wound healing (80), and are also involved in pathological conditions such as tumour metastasis (79) and cardiac remodelling (81).

In healthy tissue MMP activity normally is low, and the MMPs are tightly regulated at both transcriptional level through control of the activation of the latent enzymes, and through inhibition by specific endogenous tissue inhibitors of metalloproteinases (TIMPs) (82). During pathological conditions the zymogens can be activated by proteolytic cleavage (78), or stepwise by low pH (83), oxidative stress (84-88), and during inflammation (49;75;77). MMPs are thought to act as signalling proteinases, and activation may occur within seconds to minutes (89). Increased MMP-levels reflect illness, and during acute myocardial ischaemia and subsequent reoxygenation different MMPs are involved (65;90).

In a human study of term and preterm neonates, plasma level of MMP-2 and MMP-9 was found to be gestational dependent, with the highest levels at 33-36 weeks of gestation (91). MMP-2 and MMP-9 were lower in term neonates as in preterm babies with earlier gestational age. MMP-9 showed a 50% decrease the first postnatal month, while MMP-2 was stable in the first month after birth (91). Accordingly we would have expected steady levels of the assessed MMPs in healthy neonates not suffering from asphyxia.

Resuscitation of the newborn

According to World Health Organization resuscitation is estimated to be needed in approximately 3-7 % of newborn infants to establish a vigorous cry or regular respiration, maintain a heart rate >100 beats per minute and achieve good colour and tone (92;93). Resuscitation procedures vary worldwide from gentle stimulation to more vigorous treatment (94;95). Even though mouth-to-mouth breathing was practised hundreds of years ago by midwives to revive stillborn infants, it fell out of fashion in the 18th century as Dr Hunter, an enormously influential obstetrician, declared it “a vulgar practice” (95).

Resuscitation in the newborn is different from resuscitation in other groups of age, and knowledge of relevant physiology and pathophysiology is essential. In contrast to adults, neonates usually have a beating heart during resuscitation, though heart rate and cardiac output may be severely depressed. Bradycardia is in most cases caused by hypoxaemia (96;97), and the tendency of apnoea induced by upper airway stimulation is enhanced by hypoxaemia (98-100). Ventilatory rate is normally increased by hypoxaemia, which stimulates pulmonary stretch receptors with a concomitantly reduced vagal tone and increased heart rate (99;101). Accordingly, the major importance is to emphasize ventilation, and ensure adequate inflation of the lungs to reverse hypoxia, acidosis, and bradycardia (93;97;102;103).

Airways should be cleared and opened before ventilation is started. However, care must be taken as laryngeal suctioning and unsuccessful attempt of intubation may aggravate both the apnoea and the bradycardia (93;98). Severe asphyxia may eventually cause cardiac arrest, and cardiac compressions should be started if heart rate remains low (less than 60 beats per minute) despite adequate ventilation for 30s. The most efficient way is to encircle the chest with both hands with the thumbs opposed on sternum just below the inter-nipple line. Current recommendations are a 3:1 ratio with 90 compressions and 30 breaths per minute at a rate of 120 events per minute to achieve approximately 90 compressions and 30 breaths per minute. Drug therapy should be considered if there is no response in heart rate after 30 sec of combined ventilation and chest compression (93;97). Hypovolemia should be suspected if the neonate does not respond to resuscitation, and treatment with volume expansion may be needed. An isotonic crystalloid solution is recommended as volume expander. If replacement of large blood volume loss is necessary, O-negative red blood cells is indicated (93). Heat loss should be prevented as oxygen consumption is increased by cold stress and may impede effective resuscitation.

Until recently, 100% oxygen has been recommended during resuscitation of asphyxiated newborn infants (104). Over the last few years, however, there has been growing evidence that ambient air may be as efficient as 100% O_2 (105-107). Regarding the latest guidelines of resuscitation of newborn, the goal of supplemental oxygen use is normoxia (93;108), and ambient air resuscitation of asphyxiated neonates is recommended when availability of oxygen is limited (93;109).

Treatment strategies

Ventilation

Little evidence-based data have been available regarding optimal resuscitation methods of newborn, and questions have been raised to improve treatment strategies and performance of resuscitation (110;111). Establishment of adequate ventilation is of primary concern (93), and bag/mask-ventilation performed by the experienced is thought to be satisfactory in 80% of the resuscitated infants. Endotracheal intubation is needed in only 20% or less (112). The initial inflation of the fluid-filled lungs need higher opening pressure and inspiratory time than the subsequent breaths of the newborn (102;113). When functional residual capacity is optimised, end-expiratory airway pressure can be decreased without a great loss of lung volume as long as the pressure is not decreased below the critical closing pressure of the lung (114). Positive end expiratory pressure (PEEP) is thought to be important for neonatal ventilation. Using PEEP during resuscitation has shown to improve compliance of the respiratory system without any differences in arterial carbon dioxide tension ($PaCO_2$) (115). A PEEP level of 5 mmHg during ventilation of children demonstrated elevated cardiac index and stroke volume index compared to higher levels of PEEP (116). There are, however, still insufficient data to determine the safety and efficacy of PEEP during neonatal resuscitation (117).

Also initially, during the first positive pressure breaths administered during resuscitation, harmful ventilation may cause severe pulmonary disease in newborn as a result of 'barotrauma' (implying injury caused by pressure), 'volutrauma' (implying injury caused by excessive tidal volume delivery), and/or 'atelectrauma' (implying injury caused by alveolar collapse) (118-120). Hyperventilation in infants with persistent pulmonary hypertension is associated with poorer neurodevelopmental outcome (121). In animal models it has been

shown that hyperventilation may be detrimental during resuscitation, probably as a result of increased intrathoracic pressure causing considerably decreased coronary perfusion pressures (122;123).

Oxygen

Oxygen as a separate gas was recognized in the late 18th century, independently discovered by the Swedish pharmacist Karl W. Scheele and the English chemist Joseph Priestly. Oxygen was described as a necessary but dangerous substance, essential for multicellular life. The first experiments on humans are said to have been made by Chaussier in 1783, giving oxygen to infants suffering from asphyxia, and to dyspnoeic patients with phthisis (124). Ventilation-perfusion mismatch and diffusion impairment were described as a cause of hypoxaemia in the early 20th century (54;125;126), and administration of oxygen became routine to patients with acute and chronic lung disease observing improved lung function. Oxygen was regarded as *a medicine.* Oxygen supply for neonatal resuscitation was not recommended before the 20th century (95). Since then oxygen has been one of the most commonly used therapies in the neonatal intensive care unit (127).

Soon after its discovery, however, oxygen was acknowledged as potentially poisonous. Oxygen was considered to be "a good thing", though it was possible to have "too much of a good thing" (124). In the early 1950's the first observations regarding excess oxygen resulting in retrolental fibroplasia were reported (128;129), and investigation in oxygen has since then generated considerable information regarding both physiological and toxic effects of oxygen.

Molecular oxygen is a major source generating ROS. Mitochondrial electron transport reduces 95% of O_2 to H_2O without any free radical intermediates; the remaining 5% of

oxygen however, is reduced producing free radicals. Oxygen toxicity is mainly believed to result from ROS formation. When ROS generation is in excess of antioxidant defence mechanisms, the condition can be defined as oxidative stress. Biological cells maintain a delicate balance between the protective oxidant signalling versus detrimental effects and this balance seems a critical aspect of aerobic life (68).

Despite decades with research in both human and animal models, there is still an ongoing debate and no consensus regarding the optimal concentration of oxygen supply for neonatal resuscitation.

Aims of the study

Resuscitation after birth is a common procedure, and it is important to develop optimal resuscitation methods to prevent additional iatrogenic injury. Over the last few years, there has been growing evidence that ambient air is as efficient as 100% O_2 (105;130;131). Little attention has, however, been paid to whether ventilation frequency or modus operandi of resuscitation influences the outcome of the newborn. Myocardial function and circulation are important during and after resuscitation of the asphyxiated newborn. We therefore focused on myocardial injury and performance during hypoxaemia and subsequent reoxygenation in a model of newborn pigs subjected to severe hypoxaemia.

1) A) Is myocardial injury after hypoxaemia and subsequent reoxygenation assessable by clinically well established blood tests (cTnI, CK-MB, myoglobin) in newborn pigs? B) Does reoxygenation with 21% or 100% oxygen at different $PaCO_2$ levels or ventilatory mode during reoxygenation influence myocardial injury as assessed by cTnI, CK-MB, and myoglobin? (Paper I)

2) Does severe hypoxaemia cause haemodynamic changes in newborn pigs assessable by conventional Doppler echocardiography, and is outcome different if reoxygenation is performed with 21% or 100% oxygen? (Paper II)

3) Are there any differences in myocardial tissue injury as assessed by matrix metalloproteinases and endogenous antioxidant capacity in myocardial tissue extracts subsequent to reoxygenation with 21% or 100% oxygen at different $PaCO_2$ levels in hypoxaemic newborn pigs? (Paper III)

4) Is myocardial left ventricular performance in hypoxaemic newborn pigs assessable by strain Doppler echocardiography, and is it necessary to evaluate left ventricular function by differentiating between longitudinal and radial contraction in newborn pigs during hypoxaemia? (Paper IV)

Methodological considerations

The animal model

Animal models will always be an approximation of the clinical situation of interest. There may be important differences between the model and the clinical situation that should be kept in mind when results are interpreted. Nevertheless, animals are extensively used in biomedical research in an attempt to understand the mechanisms of disease using methods not available in clinical research. There are, however, some ethical dilemmas performing animal research, such as the importance of avoiding painful procedures of the experimental animal. The justification of taking the life of other species should always be considered when new experiments are planned. Nevertheless, when studying complex integrative physiology where the number of unknown variables influencing the responses studied is large, at present there is no alternative to animal experiments.

In the present studies newborn Landrace pigs of either sex (12-36h) were exposed to hypoxaemia by ventilation with 8% O_2 in N_2 until either mean arterial blood pressure (MAP) decreased to 15mmHg or BE ≤ -20mmol/L. Mean hypoxaemia time was 60 min. The pigs were then reoxygenated with either 21% or 100% oxygen for 30min. In paper I/III each of these major groups were divided into three subgroups during reoxygenation, and ventilated at different $PaCO_2$-levels (Fig 1). Subgroups A1/B1 were hyperventilated, $PaCO_2$ 2.0–3.5 kPa. Subgroups A2/B2 were normoventilated, $PaCO_2$ 4.5–6.0 kPa, and subgroupsA3/B3 were normoventilated and added CO_2 gas to reflect hypercapnia, $PaCO_2$ 8.0-9.5 kPa. The pigs were then observed and ventilated with ambient air and normal $PaCO_2$ 4.5–6.0 kPa.

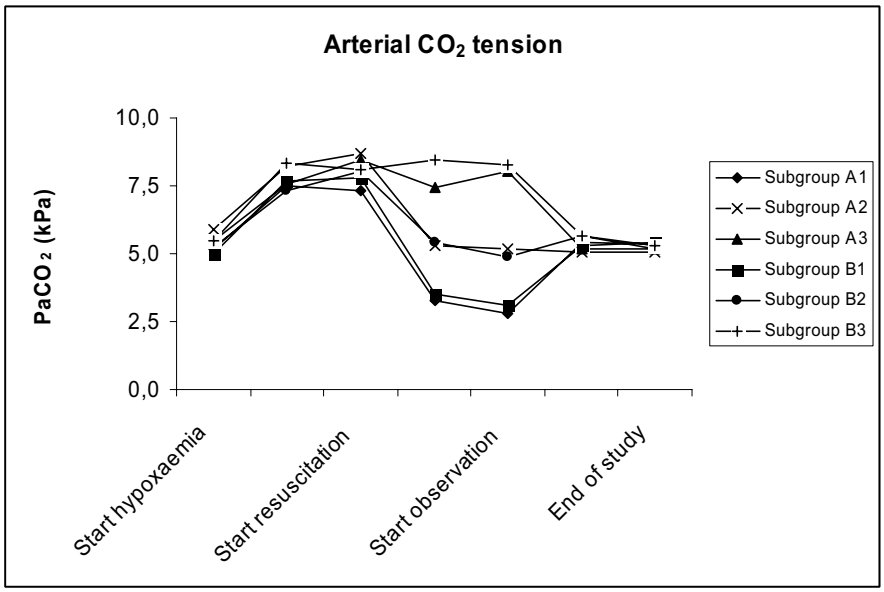

Fig 1: Time course during resuscitation.

The pig as an experimental animal

We used a porcine model, because newborn pigs resemble humans in anatomy and physiology of the cardiovascular system (132;133). In addition to the size and morphological characteristics, there are physiologic similarities in the areas of coronary blood flow (134), growth of the cardiovascular system, and neonatal pulmonary development (133).

There are, however, some differences. The porcine pericardium is attached to the sternum, and the apex of the heart is in close opposition to the diaphragm. Myocardium in newborn pigs is delicate and especially susceptible to arrhythmias. Care must be taken to avoid adrenergic stimulation by stressing the pigs (133).

Another difference that may influence haemodynamics is haemoglobin (Hb) level between human and pigs. Normal Hb in newborn babies the first day of life is approximately 18.5g/dL (135). In her thesis Egeli found mean Hb 7.9 g/dL in newborn pigs (136), and in newborn pigs there has been found an inverse relationship between CO and Hb levels (132). At birth human infants mostly have fetal Hb, while Hb in newborn pigs resembles adult Hb (137). The lower Hb level may partly be compensated for by a higher oxygen extraction fraction. In our experiment there were no differences between the groups examined and compared. The newborn pigs examined in paper I/III had Hb 7.7 g/dL (± 1.5), and in paper II/IV Hb was 6.8 g/dL (± 1.2). These are slightly beneath normal values in newborn pigs, and there were no difference in Hb between groups of pigs compared in the thesis.

Anaesthesia

The use of anaesthesia is a problem in animal research, as it will affect the results, complicating interpretation and comparison of previous experiments. There is, however, unethical to perform animal experiments without adequate anaesthesia. On the other hand, pigs are easily stressed, and pain and anxiety in the experimental animals would also affect the results by adrenergic stimulation and not reflect the true resting situation.

In the present work anaesthesia was induced by halothane mixed with ambient air and oxygen, and was discontinued when an ear vein was cannulated usually within two-three minutes. Fentanyl, Midazolam, and pentobarbital sodium was then titrated and given as bolus injections. Local infiltration of lidocain in the skin was given before tracheostomy was performed, and anaesthesia was thereafter given as a continuous infusion of fentanyl 25-50 µg/kg/hour and midazolam 0.25mg/kg/hour. One bolus of pancuronium was given before the

surgical preparations. Depth of anaesthesia was monitored by response to painful stimuli elicited by pinching of the nasal septum in addition to standard monitoring of heart rate and blood pressure. Additional fentanyl was given if necessary. At the end of the experiment, the pigs were given an overdose of 150 mg/kg pentobarbital intravenously.

In the present work we tried to use anaesthetics recommended and preferred for cardiac procedures (133;138). The medication used to induce anaesthesia, however, has dose-dependent effects on the cardiovascular system. Both halothane and pentobarbital were given in low doses, and was usually discontinued for approximately two hours prior to the experiment as a consequence of time for instrumentation and stabilisation, thus we regard the effect of these medications to be minor or ignored. The effects of halothane are dose related and dose dependent. Halothane has a potential cardiovascular depressive effect, and may

depress myocardial contractility and myocardial metabolic activity as a result of inhibition of glucose uptake in myocardial cells. Halothane may reduce cardiac output (CO) and decrease mean arterial blood pressure, while it causes no change in systemic vascular resistance. Arrhythmias may occur, both bradyarrhythmias caused by central vagal stimulation and tachyarrhythmias associated with increased excitability augmented by hypercapnia, hypoxaemia or circulating catecholamines (133;138).

Pentobarbital 50mg/ml was titrated in doses of 10mg until tracheostomy was performed, and then discontinued. Pentobarbital is recommended used as pig anaesthesia, though it may cause respiratory depression, decreases myocardial contractility and CO, and increases peripheral vascular resistance (133).

Fentanyl is an opioid and has minimal cardiovascular effects. Fentanyl has no direct effects on heart, hence there is no change in contractility, automaticity (though increased vagal activity), conduction, or sensitivity to catecholamines. Bloodflow autoregulation in heart is preserved. Combined with other hypnotica bradycardia or hypotension may occur (133;138).

Midazolam is a benzodiazepine with mild cardiovascular effects. Midazolam may slightly reduce systemic arterial blood pressure, and to a lesser degree decrease cathecolamin release and increase coronary blood flow. Combination of benzodiazepines and opioids are recommended for cardiac investigation. If combined with opioids in larger doses, however, midazolam may cause cardiodepression (133;138).

Pancuronium is a muscle relaxation given only once as a bolus of 0.1mg/kg before the initial surgical procedures. Pancuronium has a slight effect on cardiovascular system (133;138).

Surgical setup and invasive pressures

The experimental animals were exposed to miner surgery, but it may nevertheless influence outcome. Both tracheostomy and insertion of catheter in the femoral artery measuring blood pressure occasionally injured striated muscle, which probably affected the blood tests (CK-MB, myoglobin). In the pigs referred to in papers II /IV there were inserted a catheter for measuring central venous pressure in left external jugular vein, and a Millar catheter in LV through the left carotid artery, assessing left ventricular pressure (LVP) and calculating LV dP/dt from the LVP curves. The Millar catheter is known to be prone to drifting. This is, however, a result of protein deposits or a film covering the micro manometer pressure sensor of the catheter. To minimise drift, the catheter was cleaned immediately after us, the pressure sensor was pre-soaked in tempered (38°C) saline for 30 minutes prior to calibration, and the catheter was used as recommended by the manufacturer (139).

Though we allowed a period of recovery after the initial procedures and care was taken to minimise myocardial damage, baseline measurements may probably be impaired compared to healthy non-instrumented pigs.

Blood and tissue analyses

CTnI, CK-MB, myoglobin

Cardiac Troponin I (cTnI) has been established as a sensitive and specific marker of myocardial injury both in humans and pigs (140-142), with no increase in serum subsequent to acute or chronic skeletal muscular disease (143). In human neonates, cardiac troponin is unaffected by gestational age, birth weight, sex and mode of delivery (140;144). CTnI has been suggested as a new “golden standard” in paediatric patients (145), even though

comparing measured cTnI values between different studies are difficult because of variations in sensitivity and specificity of commercially available cTnI diagnostic immunoassays kits (146-148). In the thesis the analyses of cTnI were performed by two different immunoassays, none of them cross reacting with cardiac troponin T or slow skeletal muscle troponin I. The different immunoassays, however, implies that cTnI values in paper I/III and paper II/IV are not directly comparable.

CK-MB (creatine kinase-myocardial band) has high specificity and was earlier used as "gold standard" for the diagnosis of myocardial infarction. Soldin et al found CK-MB proteins more pronounced in healthy term neonates than in adults (149), and during fetal and neonatal development, the B-subunit is found to be the predominant CK species produced by skeletal muscle (143). CK-MB has been used as an early (<6 hours) marker of acute (myocardial) muscular injury (150). Myoglobin is predominantly a cytosolic protein found in striated muscle, and there is no difference between isoforms found in myocardium compared to skeletal muscle (151). Several authors have found myoglobin and CK-MB as less sensitive and specific markers of myocardial injury than troponin (149;152;153) which is consistent with our results (154).

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are markers of myocardial tissue injury reflecting illness (65;155). Myocardial MMP-activity has been correlated with cardiac functions in humans (52), and inhibition of MMP-activity has been shown to significantly improve cardiac functions during reperfusion subsequent to ischaemia (156). During acute myocardial ischaemia and subsequent reoxygenation different MMPs are involved (90). MMP-1, 2, and 9 appear to be of especially importance as they are differently generated and most likely

responsible for alternative aspects of the pathological processes in myocardium (65). Raised MMP-1 levels secondary to myocardial infarction is thought to predict subacute LV remodelling (157), while enhanced levels of MMP-2 and 9 have been associated with reperfusion injury after ischaemia contributing to cardiac mechanical dysfunction in different experimental models (158-160). MMP-2 has demonstrated intracellular activity by cleaving cTnI from the troponin complex (156), and may also be released by platelets activated during ischaemia (89;161). Thus MMP-2 may contribute to myocardial cellular injury as well as impaired myocardial contractility. Recently in a resuscitation model of hypoxaemic newborn pigs (162) MMP-2 was found increased and probably activated because of oxidative stress, in agreement with observations in our study (163).

In the previous study, we have analysed MMPs in myocardial tissue extracts by two different methods. Broad matrix degrading capacity is the analysis of the joint capacity of MMP-1, MMP-2 MMP-7, MMP-8, MMP-9, MMP-12, and MMP-13 referred to as "total MMP activity", using a fluorogenic peptide substrate. In gelatine zymography, the MMPs are identified by the capacity of degrading gelatine after electrophoresis. The MMPs are identified by molecular weight. MMP activity is detected as clear bands against a background of undegraded substrate. Incubation with EDTA was used as a control demonstrating that the metal-dependent lysis zones were the result of gelatinase activity. One tissue sample and a human standard were used as internal controls on every zymogram.

Endogenous antioxidant capacity

The oxygen radical absorbance capacity (ORAC) assay measures free radical scavenging activity against ROS in tissue samples, the total antioxidant capacity. Antioxidant capacity is defined as the ability of a compound to reduce pro-oxidants. Several compounds are

considered to act in a concerted way providing antioxidant defences to the organism, protecting against harmful effects of ROS (64;164). There are also several combinations of internal and external antioxidants and the radicals they combat. This might explain why no single measure of antioxidant status is going to provide a sufficient amount of data to evaluate in one assay the free radical scavenging activity of a tissue sample.

The ORAC assay utilises a biological relevant radical source and measures the protective effect of antioxidants as the capacity of the tissue sample to directly quench free radicals. Trolox, a water-soluble vitamin E analog, is used as the calibration standard and the ORAC result is expressed as micromole Trolox equivalent (TE) per gram tissue.

ORAC is the only method combining both percentage degree of inhibition and inhibition time of free radical action by antioxidants into a single quantity (74). The ORAC assay has high specificity, and provides significant information regarding the antioxidant capacity of biological samples (165;166). The ORAC method is sensitive and has several advantages in comparison to other commonly used methods, as it gives more information of the in vivo situation in tissues (74). Regardless of the source of oxygen-derived free radicals, cellular injury may occur during reperfusion if the level of oxidative stress exceeds the capacity of endogenous free radical scavenging mechanisms (167). As the total endogenous antioxidant capacity decreases, the protective mechanisms against oxygen toxicity is reduced (168), thus describing a considerable creation and consumption of ROS implying oxidative stress in the tissues (84;168;169).

Doppler echocardiography

The Austrian physicist Christian Johann Doppler first hypothesised “the Doppler effect” in 1842, the phenomenon when a source and receiver are moving relative to each other, and the perceived frequency of any kind of wave is altered. For measuring blood flow velocity, an ultrasound transducer is both the wave source and receiver, whereas red blood cells are the moving targets that alter or “shift” the received frequency depending on their velocity and direction (170;171). Ultrasound is acoustic waves with a frequency above the audible range. For diagnostic purposes, frequencies in the range of 1-15 MHz are used. The first use of a Doppler method for detection of cardiac motion was described by Yoshida et al (172). Technology has improved considerably over the last decades, and today Doppler ultrasound and two-dimensional echocardiography are the main tools in routine investigation and follow-up newborn with circulatory disturbances and/or congenital heart disease (171;173).

We used a Vivid 7 digital ultrasound scanner (GE VingMed Ultrasound, Horten, Norway) with an integrated program for quantitative analysis (software version 2.2.0 EchoPAC GE Vingmed). The transducer was a combined tissue imaging and Doppler transducer (10S) with a frequency range from 4.0 to 12.0 MHz. The recordings were mainly obtained using the factory default 8.9 MHz. Hair in front of the chest was removed prior to the examinations. Two investigators, one handling the probe and one operating the scanner performed all ultrasound standard examinations (132;174). The data were sampled when both investigators agreed that the imaging quality was optimal. The accuracy of Doppler velocimetry is affected by the angle between the ultrasound beam and blood flow, thus for each view care was taken to keep the Doppler beam oriented as parallel as possible to the direction of motion (175).

The first examination was performed to exclude congenital heart defects with the pigs lying in a right lateral, half supine position. The aortic valve diameter was measured from 2D parasternal long-axis views from inner edge to inner edge at the base of the aortic valve leaflets (176) before guiding the intrusion of the Millar catheter. The aortic valve diameter was later used for all calculations of cardiac output (CO). Further studies were performed with the pigs lying in a left lateral, half supine position. CO was calculated from aortic flow as systolic velocity integral x valve area x heart rate. All ultrasound measurements used in the final calculations for each pig were mean values, automatically calculated in the ultrasound instrument, from three to five repeated selected sequential beats regarded as good quality measurements (174). The peak tricuspid regurgitation velocity (TR-Vmax, m/sec) was sampled from apical "four-chamber view" to calculate the pressure gradient between the right ventricle and the right atrium (mm Hg) by using the modified Bernoulli's equation (177). Systolic right ventricular pressure (RVP) was calculated indirectly as the sum of central venous pressure and the pressure gradient between the right ventricle and the right atrium. Different methods for estimating pulmonary artery pressure are currently available. The pulmonary artery systolic pressure estimation from the peak velocity of the tricuspid regurgitation is evaluated to be the most precise non-invasive method (177;178).

Tissue Doppler imaging (TDI)

Traditionally Doppler ultrasound has been applied for measuring blood flow velocities. In contrast to blood flow reflecting high velocity and low amplitude Doppler signals, myocardial tissue movements produce low velocity and very high amplitude Doppler signals. Blood flow imaging applies a high-pass filter incorporated to eliminate the strong and low-frequent tissue signals and the gain settings are increased to amplify the signals estimating blood velocity (179). To display tissue velocities, the high-pass filter is bypassed and a lower gain

application is used to eliminate the weaker intensity blood flow signals (179). The technical principles and limitations of TDI are similar to those encountered with conventional Doppler echocardiography (179). Angle dependency is an important limitation, and angles < 16 % has been regarded as acceptable giving less than 20 % error (180-182). Aliasing is another well known phenomenon in Doppler echocardiography, and when the velocity exceeds the maximum detectable frequency (the Nyquist limit), the velocities appear to be transposed to the other end of the scale (171;182). The interplay between frame rate and temporal filtering must be considered, and to avoid the risk of loosing information of myocardial motion it may be preferred to use high frame rates and filtering (183).

TDI was introduced as a method to quantify myocardial function in terms of tissue velocity (183-185).The capability to distinguish local velocities from translational motion and tethering effects from other regions, are however impossible using tissue velocity. Thus to improve the ability of TDI to measure regional function, Heimdal et al. introduced cardiac *real time strain* and *strain rate* as a novel extension of TDI (186).

Strain Doppler echocardiography (SDE)

The concept of myocardial strain was defined by Mirsky et al (187) as fractional tissue deformation in response to applied force (stress). In this context strain is directly related to myocardial fibre shortening, and represents fractional change of tissue length (change in length per unit length). Strain reflects deformation of myocardium and is expressed as percent shortening or lengthening (Lagrangian formula).

In general terms, if an object has an initial length L_0, and after a certain time changes to the length L, strain is defined as

$$\text{strain} = \frac{L - L_0}{L_0}$$

Thus strain describes the contraction/relaxation pattern in the myocardium, and both longitudinal and radial strain may be assessed by this Doppler technique (188). Strain represents a powerful non-invasive diagnostic tool to assess LV function (189). Strain has been shown to be superior to tissue Doppler velocity for measuring systolic function (190;191) and the modalities are less sensitive to segment tethering and cardiac translation (192). Analyses of strain appear to be less prone to noise than strain rate because integration from strain rate tends to minimize the influence of small random changes (193). By current methods SDE can only be assessed one-dimensional along the ultrasound beam. Despite the limitation, SDE has been reported to correlate well with 3D magnetic resonance (MR) findings (194) as well as to sonomicrometry in animal models (195). It has been shown that strain representing deformation of myocardium is load dependent and is best correlated with stroke volume (196) and ejection fraction (197).

During the experiments the strain images were obtained with a frame rate varying between 180-250 frames per second. Digital data were recorded over 3 consecutive heart cycles and analysed off-line. Standard apical 2-chamber and 4-chamber views and parasternal short axis views were used. To maintain a high frame rate without loss of lateral resolution, separate loops were obtained to focus on septum and lateral wall. Sample volume was set to 5 mm for longitudinal measurement and 2 mm for radial measurement. During off line analysis, longitudinal peak strain was assessed at mid anterior, posterior, lateral, and septal segments.

In short axis views radial peak strain values were assessed in the anterior septum and posterior wall segments. The strain data were calculated and averaged from all three cardiac cycles. The region of interest in the 2D-image was repositioned during the heart cycle by manual frame to frame marking to compensate for the movement of the heart. This corrected strain trace was used for processing. The interventricular septum is believed to be a functionally bilayered structure, with the presence of two radial velocity gradients within the septum, one on the LV side and one on the right (182;198). The sample volume was set to 2 mm, with no possibility to differentiate between left and right part of the septum in the newborn pig.

Summary of results

Paper I

Resuscitation with 100% O_2 does not protect the myocardium in hypoxic newborn piglets

58 newborn pigs (12-36h) divided into a total of 6 groups (n=9/10) were exposed to hypoxaemia by 8% oxygen. When mean arterial blood pressure fell to 15 mmHg, or arterial BE ≤ -20 mmol/L, reoxygenation was performed for 30 minutes with either 21% or 100% oxygen at low ($PaCO_2$ of 2.0–3.5 kPa), normal ($PaCO_2$ of 4.5–6.0 kPa) or elevated ($PaCO_2$ of 8.0–9.5 kPa) carbon dioxide level. Afterwards the pigs were ventilated with 21% O_2 and normal $PaCO_2$ for 150 minutes. Blood samples were analysed for cTnI, myoglobin, and CK-MB at baseline and at the end of the study. CTnI increased more than 10-fold ($p < 0.001$) in all the groups, with no significant difference between the groups. Myoglobin and CK-MB doubled in concentration. The considerable increase in cTnI indicated a substantially affected myocardium due to severe hypoxaemia. Reoxygenation with 100% oxygen offered no biochemical benefit over ambient air. CK-MB and myoglobin were not reliable markers of myocardial damage in this model of newborn pigs.

Paper II

Restoration of cardio-pulmonary functions with 21% vs. 100% O_2 after hypoxaemia in newborn pigs

20 newborn pigs were exposed to hypoxaemia by 8% oxygen. When mean arterial blood pressure fell to 15 mmHg, or arterial BE ≤ -20 mmol/L, reoxygenation was performed with either 21% (n=10) or 100% (n=10) O_2 for 30 min and then ventilated with ambient air for another 120 minutes. Blood was analysed for cTnI, confirming a considerable myocardial injury with no differences between pigs reoxygenated with 21% and 100% O_2. Ultrasound examinations of cardiac output (CO) and pulmonary artery pressure (PAP, estimated from tricuspid regurgitation velocity (TR-Vmax)) were performed at baseline, during hypoxaemia, at start of reoxygenation and during reoxygenation. TR-Vmax increased during the insult, returned towards baseline values during reoxygenation, with no differences between the groups (p=0.11). An inverse relationship was found with increasing age and TR-Vmax during hypoxaemia (p=0.034). CO/kg increased during early phase of hypoxaemia (p<0.001), then decreased. Changes in CO/kg were mainly due to changes in heart rate with no differences between the groups during reoxygenation (p=0.298). Global hypoxaemia affects myocardium and pulmonary artery pressure in newborn pigs and during this limited period of observation, reoxygenation with 100% showed no benefits compared to 21% O_2 in normalising myocardial function with respect to CO and pulmonary artery pressure.

Paper III

Increased Myocardial Matrix Metalloproteinases in Hypoxic Newborn Pigs during Resuscitation. Effects of Oxygen and Carbon Dioxide

At the end of study referred to in paper I the pigs were given an overdose of penthobarbital, and the heart was immediately removed. The free wall of left and right ventricle was instantly frozen in liquid nitrogen. In myocardial tissue extracts, MMPs were analysed by *i*) gelatine zymography and *ii*) broad matrix degrading capacity (total MMP-activity). Total endogenous antioxidant capacity in myocardial tissue extracts was measured by the oxygen radical absorbance capacity (ORAC) assay. As a sign of affected myocardium MMP-2 more than doubled from baseline values ($p<0.001$), and was more pronounced in piglets reoxygenated with 100% than with 21% O_2 ($p=0.01$). The ORAC value was considerably decreased ($p<0.001$), suggesting that oxidative stress may be a key player causing adverse tissue injury. Left ventricular (LV) total MMP-activity was increased in pigs with low $PaCO_2$ compared to pigs with elevated $PaCO_2$ ($p=0.01$), indicating that hyperventilation may be detrimental and cause more severe myocardial injury. LV total MMP-activity in pigs with elevated $PaCO_2$ was not different from baseline. In pigs with elevated $PaCO_2$, total MMP-activity was more increased in right (RV) than in left ventricle ($p<0.01$). Alteration in total MMP-activity subsequent to hypoxaemia and resuscitation, suggests that elevated carbon dioxide ($PaCO_2$ of 8.0–9.5 kPa) may protect LV, and contribute to a greater RV injury in newborn pigs. This study demonstrates the importance of resuscitation procedures, since not only oxygen supply but also the $PaCO_2$-level has to be addressed to avoid additional iatrogenic tissue injury during resuscitation of neonatal pigs.

Paper IV

Left Ventricular Longitudinal Deformation is More Vulnerable than Radial Deformation. A Strain Doppler Echocardiographic Study in Hypoxaemic Newborn Pigs

11 newborn pigs (age 12 to 36 hours) were exposed to hypoxaemia by 8% oxygen. When mean arterial blood pressure fell to 15 mmHg, or arterial BE ≤ -20 mmol/L, reoxygenation was performed with 21% or 100% oxygen and the pigs were observed for 150 min. Analyses of the two groups have so far been combined. Cardiac dysfunction during global hypoxaemia was assessed by strain Doppler echocardiography (SDE) which represents a powerful non-invasive diagnostic tool to assess LV function. During hypoxaemia longitudinal strain showed paradoxical stretching, while radial strain continued to demonstrate thickening. In addition, the changes in the longitudinal myocardial contraction were heterogeneous. These findings suggest that longitudinal contraction may be more vulnerable to global hypoxaemia than radial contraction in newborn pigs. Accordingly, in cases when radial functions appear conserved, longitudinal strain assessments may be necessary to exclude hypoxaemic injury of the myocardium

General discussion

In our department we have been working with brain and lung injury subsequent to resuscitation of newborn pigs over several years, continuously trying to improve the model approaching the question whether newborn in general should be resuscitated with or without supplemental oxygen (199-203). Through induction of severe hypoxaemia we have aimed at simulating the clinical situation of intrapartum asphyxia with development of acidosis, resulting in increased PAP and myocardial dysfunction.

In this thesis we approached the myocardial injury of hypoxaemia and reoxygenation by four different methods in a model of newborn pigs. In the first and second study we were evaluating the myocardial injury by troponins and echocardiography. These are global signs of an affected myocardium, and are methods currently used in modern neonatal care units. In paper III we examined the myocardial injury directly in tissue extracts, aiming to clarify the myocardial tissue injury emphasizing both ventilation and oxygenation. In paper IV we intended to focus on LV performance and the diagnostic feasibilities of SDE, as invasive data are not always available in a clinical practise. Strain assessments and analyses of MMPs in myocardial tissue are focal markers of myocardial injury. Accordingly, we have focused on both biochemical and functional changes in myocardium during hypoxaemia and reoxygenation. The study aimed at approaching the fine balance of both physiological and molecular biological alterations in the myocardium. The problem resolving hypoxaemia-induced myocardial and haemodynamic changes in theses group of age necessitates alternative considerations.

The pig model

A limitation regarding the pig model was the age of the experimental animal, as the included pigs were 12-36 hours of age. The animals had been adapted to extra-uterine life, and had already started and more or less completed the haemodynamic changes from a dominating high pressure RV and low pressure LV system to an adult circulatory pattern with low pressure RV and high pressure LV, and with no flow through the fetal shunts (68;132). Walthers et al found that 8 hours after birth the majority of measurable changes in cardiopulmonary hemodynamics have occurred in healthy infants, though there was found some degree of right-to-left shunting in ductus arteriosus in the neonates up to 12 hours after birth (45). Fugelseth et al found bidirectional shunting in approximately 50% of the patients with an open duct at 14 hours postnatal, and mainly left to right ductal flow by 36 hours (38). This may be important as we found that age was inversely correlated to pulmonary artery pressure as assessed by TR-Vmax. Furthermore, we observed that hypoxaemia-induced haemodynamic changes were tolerated for a longer time course by the youngest pigs.

All manipulation and instrumentation (anaesthesia, surgery, hypoxaemia) were the same in both test animals and control animals, and care was take to minimize injury during procedures. The pigs went through a severe hypoxaemic insult, with grave acidosis with pH below 7,0 and serum-lactate 15 mmol/L (unpublished data) at start of resuscitation. The pigs were able to compensate with increased HR up to 270 beats per minute, before some of them eventually developed bradycardia (HR 35-70 beats per minute) with a severely compromised myocardium when resuscitation was about to start.

As demonstrated in paper IV, the restoration of myocardial function and haemodynamics are initiated but not completed by 2.5 hours. We would have needed a longer time of observation

to restore both the haemodynamic changes as well as myocardial performance assessed by SDE. We would also have needed either a larger experimental group or a longer period of observation to find out whether there would have been any differences in myocardial performance due to different reoxygenation regimens.

There are notable interindividual variations amongst the pigs, implying the need of relatively large experimental groups as they respond differently to asphyxia and need different time course for restoration. This limited time course also exclude us from studying long time effects of hypoxaemia and reoxygenation on myocardium, which would have necessitated a different study design and long term survival.

Cardiac Troponin I / MMPs

In the experiment we used cTnI as a marker of myocardial injury before and after a hypoxaemic insult. In addition to cardiac Troponin I and T, it has been shown that slow skeletal muscle troponin I is found in neonatal myocardium up to 9 months of age (28). Acidosis has a negative inotropic effect, as low pH decreases the Ca^{2+} sensitivity to the troponin complex (204). During acidosis neonatal myofibrils has shown to be more resistant to low pH than adult myocardium (28;205). Fetal myofilaments, containing slow skeletal muscle troponin I are suggested to have greater maximal force, greater maximal ATPase activity, lower minimal ATPase activity, and greater Ca^{2+} sensitivity of force development when compared with adult troponin (206). The slow skeletal muscle troponin I is probably a major contributor to preserve contractility in severely acidotic neonates.

In paper I we found that normoventilation tended to produce better myocardial outcome than hyperventilation and hypoventilation as assessed by cTnI. CTnI was measured in serum as a

global marker of myocardial injury, however, and not in myocardial tissue. In paper III total MMP-activity assessed as a focal marker of injury in myocardial tissue extracts made us conclude differently. The free wall of left and right ventricle had been analyzed separately. The major difference between cTnI in serum and MMP assessments in myocardial tissue extracts were seen in the pigs with increased $PaCO_2$-level. These pigs had been normoventilated adding CO_2 in the inspired gas to reflect hypoventilation. Total MMP-activity in LV were not increased from baseline, indicating that CO_2 may have a protective effect on LV. Contrary to the findings in LV, there were a considerably increased total MMP-activity in RV tissue extracts, probably responsible for the increased cTnI as MMP-2 cleaves cTnI from the Troponin complex (156). Low pH and elevated $PaCO_2$ may further augment the hypoxaemic pulmonary vascular resistance (207;208), suggesting increased RV workload due to an additional increase in pulmonary artery pressure at $PaCO_2$ 8.0–9.5 kPa.

Contrary to the results in paper I, in paper III we also found significant differences in myocardial tissue injury dependent on oxygen supply during reoxygenation subsequent to hypoxaemia. The highly increased MMP-2 activity in pigs reoxygenated with 100% oxygen indicates a more severe myocardial tissue injury compared to the pigs reoxygenated with ambient air. This is in agreement with a clinical study presented at Pediatric Academic Societies' Annual Meeting 2005 regarding resuscitation of asphyxiated neonates (209). In this study the newborn resuscitated with ambient air had better outcome, lower cTnI levels, less pronounced and shorter-lasting oxidative stress than newborn resuscitated with 100% oxygen.

MMPs / ORAC

In this study, the total endogenous antioxidant capacity in myocardial tissue extracts was assessed by the ORAC assay. The ORAC-value was considerably decreased from baseline, most pronounced in pigs reoxygenated by 100% oxygen. Several studies have shown that during hypoxaemia/reoxygenation/reperfusion injury, oxidative stress and ROS formation increases MMP levels in tissues (210;211). The different outcome regarding $PaCO_2$-level and ventilation were seen analysing total MMP activity which reflects the joint capacity of different MMPs. By measurements of total MMP activity we are not able to differentiate which MMPs are activated during the analysis. From other studies, however, it has been shown that several MMPs including MMP-1 and MMP-2, have been activated by oxidative stress and pro-inflammatory cytokines as TNF-α and interleukins (49;52;75). These cytokines may activate leucocytes to generate ROS resulting in myocardial injury (75;77). These results indicate that both MMP-2 activity and total MMP activity in tissue extracts probably is induced by oxidative stress, which is consistent with other studies (65;76;86;211). In our research group, in the same pig model, we found increased MMP activity and interleukin-8 concentration in pulmonary tissues (212). MMP activity and interlucin-8 were more pronounced in pigs resuscitated with 100% oxygen than in pigs resuscitated with ambient air, suggesting a marked proinflammatory response in pulmonary tissues.

Haemodynamics in the newborn

In fetal life hypoxaemia is a physiological stimulus maintaining the pulmonary vasculature constricted (26). In the pulmonary vasculature, increased oxygen is a powerful stimuli inducing vasorelaxation at birth (213). Sustained exposure to hypoxaemia leads to

vasoconstriction which is reversible by reoxygenation (53). The pulmonary vasculature is especially vulnerable to hypoxaemic stimuli during the first days of life (29;53;214). Accordingly, we found that increased PAP was age dependant, correlating inversely with age.

Concomitantly with the induction of hypoxaemia, systemic blood pressure (SBP) fell, while HR and CO/kg increased. Increased PAP or elevated PVR results in increased RV afterload. With elevated PAP, the septum shifts in a leftward direction, impairing LV ejection (215;216). Increased PAP will also reduce LV preload, further decreasing LV stroke volume. SBP and perfusion depend on the LV preload requiring a normal PVR to allow effective filling. The reduced LV preload resulting in systemic hypotension, will again affect RV function by reduced coronary perfusion pressure (217;218).

Neonates regulate their CO mainly by regulating heart rate, which is consistent with our results (29). A reduction in CO will be compensated by a redistribution of the blood flow to the various organs to maintain the SBP and adequate perfusion pressures to vital vascular beds. Changes in SBP may reflect changes in CO and/or systemic vascular resistance, and decreased LV afterload may for a short while improve the myocardial performance, resulting in reduced SBP.

In our study we found LVP changed in the same direction as CO/kg and inversely with TR-Vmax. The considerable increase in RVP exceeding LVP may contribute in reducing coronary perfusion resulting in RV ischaemia, further compromising myocardium (58;218). Low pH and hypercapnia may aggravate hypoxic pulmonary vasoconstriction (207;208). We had no echocardiographic measurements assessing neither tricuspid regurgitation nor blood flow through the fetal shunts in the pigs ventilated with elevated CO_2. Following a more

aggravated pulmonary vasoconstriction, however, we might have expected a greater tricuspid regurgitation and possibly increased ductal shunting. Severe pulmonary hypertension may cause bidirectional or right-to-left shunting in the ductus arteriosus, and may also be assessed as changed flow pattern in ductus venosus (219).

In the pigs examined by echocardiography, only one had a persistent ductus arteriosus as we started the experiment. One pig reopened the ductus arteriosus during hypoxaemia, and two reopened the duct at the end of the reoxygenation period. All ductal shunting were left-right, although two pigs had a bidirectional shunt for a short period during hypoxaemia. The maximum systolic peak velocities were not measured as the angle errors were too large. None of the pigs had a pure right-left shunt, which further could have compromised the situation.

In paper IV we focused on myocardial performance during hypoxaemia, finding a complex and heterogeneous systolic pattern with distinct regional differences as assessed by strain Doppler echocardiography. Radial systolic strains remained positive showing thickening during hypoxemia, but maximum values were decreased and delayed. In contrast, longitudinal systolic strain became positive showing a paradox lengthening in the septal and posterior segments. In the anterior and lateral segments, however, peak longitudinal strain values were decreased but remained negative. We constructed pressure-strain loops, loop area representing myocardial work. Counterclockwise rotation represented active work during longitudinal contraction, while clockwise rotation represented active work during radial contracting. During severe hypoxaemia the longitudinal strain-pressure loops orientation turned from counterclockwise to clockwise and from active to passive work in septum and posterior wall, which is in agreement with other studies (220;221). During severe hypoxaemia radial pressure-strain loops remained a clockwise rotation, indicating a still maintained active

contraction. This indicates that the direction of the pressure-strain loops contribute information regarding myocardial performance. Accordingly, longitudinal contraction may be more vulnerable to hypoxaemic insults, and radial contraction performing the major work during severe hypoxaemic conditions in newborn pigs.

Conclusion

1) Cardiac Troponin I is shown as a reliable blood test demonstrating myocardial injury in hypoxaemic newborn pigs. CK-MB and myoglobin were not reliable markers of myocardial damage. Reoxygenation with 100% oxygen at different $PaCO_2$ levels offered no biochemical benefit over reoxygenation with ambient air.

2) Hypoxaemia decreases cardiac output and increases pulmonary artery pressure in newborn pigs. Reoxygenation with 100% oxygen showed no benefit compared to 21% oxygen in restoring haemodynamics.

3) Matrix metalloproteinases (MMPs) were considerably increased indicating myocardial tissue injury and more pronounced after reoxygenation with 100% than 21% oxygen. As assessed by MMP activity, hyperventilation and low $PaCO_2$ may be detrimental to myocardium. Elevated $PaCO_2$ during resuscitation may protect left ventricle and probably increase right ventricular injury. Antioxidant capacity was decreased indicating oxidative stress, but with no differences between the reoxygenation groups.

4) Rapid changes in left ventricular performance during hypoxaemia and reoxygenation in newborn pigs are assessable by strain Doppler echocardiography. Longitudinal contraction seems to be more vulnerable to hypoxaemia than radial contraction; accordingly both longitudinal and radial functions have to be considered during a hypoxaemic injury.

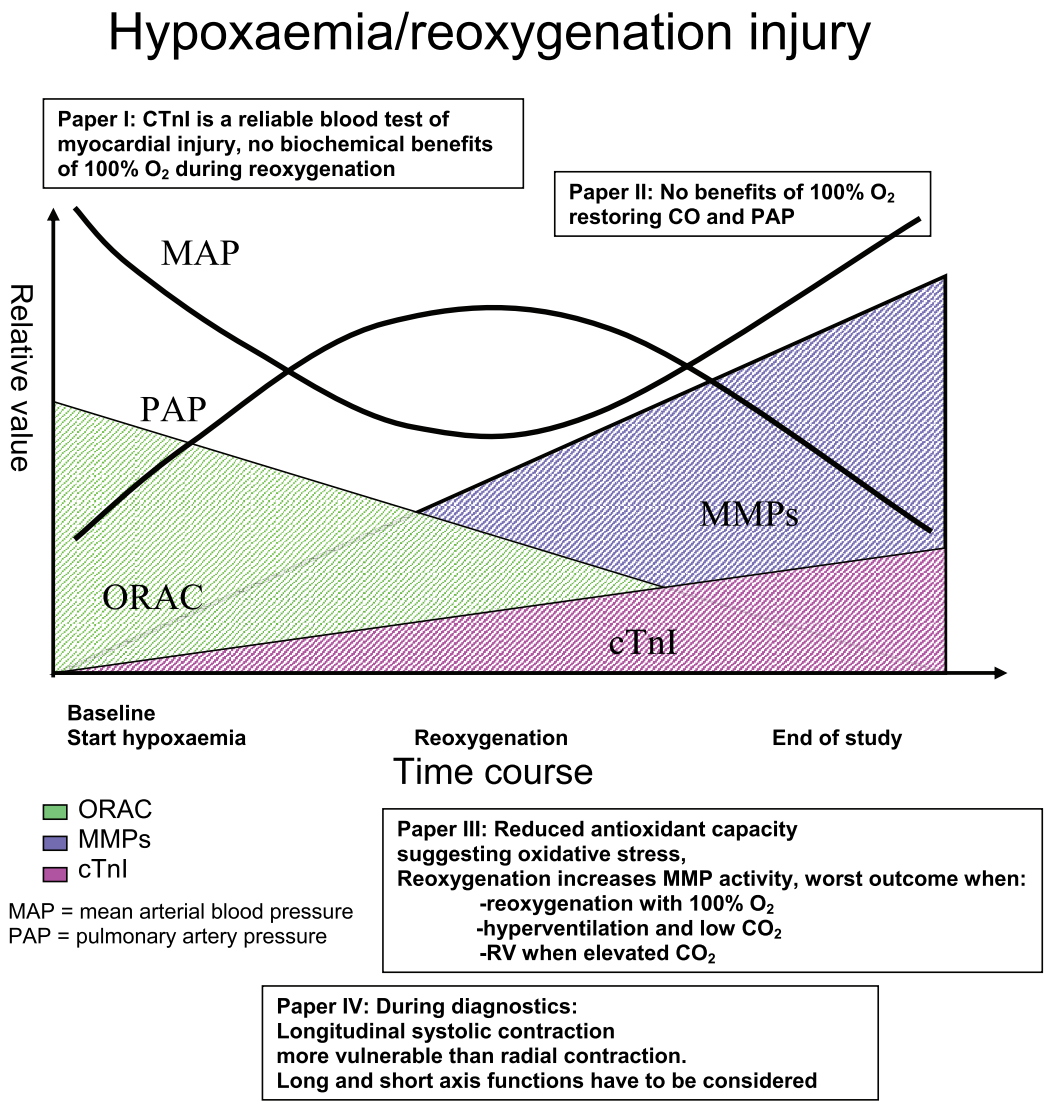

Figure 2: Short summary of the papers included in the thesis and their relation to diagnosis or myocardial outcome of hypoxaemia and reoxygenation.

This study emphasizes the importance of resuscitation procedures, since not only oxygen supply but also ventilation and $PaCO_2$-level has to be considered to avoid additional iatrogenic tissue injury during resuscitation of newborn pigs.

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Errata

Paper III

Results

Second column, **Gelatinolytic activity in heart extracts**, eighth line should read;

“MMP-2 was significantly higher in group B than in group A (p=0.012).

Second column, **Total MMP activity**, fourth line should read;

“Left ventricle total MMP-activity increased in group A1/B1 (14930.0 R.F.U. ± 922, $P = 0.003$) and group A2/B2 (13634.5 R.F.U. ± 1240, $P = 0.027$) compared to the control piglets (9606.5 R.F.U. ± 1453)”.